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No evidence for a relationship between hemolymph ecdysteroid levels and female reproductive behavior in *Schizocosa* wolf spiders

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Abstract. This study used radioimmunoassay (RIA) to explore the relationship between levels of hemolymph ecdysteroids and female reproductive behavior in *Schizocosa* wolf spiders. Specifically, we investigated the relationship between circulating ecdysteroid concentrations in females and 1) likelihood to copulate, or female receptivity [Experiment 1— *Schizocosa avida* (Walckenaer 1837)], 2) time post copulation (Experiment 2— *Schizocosa rovneri* Uetz & Dondale 1979) and 3) exposure to conspecific male courtship (Experiment 3— *Schizocosa uetzi* Stratton 1997). In Experiment 1, we expected higher levels of circulating ecdysteroids in receptive versus unreceptive females, based upon prior research demonstrating an increase in receptivity following injections of 20-hydroxyedysone (e.g., *Tegenaria atrica* C.L. Koch 1843). In contrast, we found no relationship between female receptivity and ecdysteroid levels. Our second experiment compared ecdysteroid levels in mated versus virgin females at three time points following mating trials (24 h, 7 d and 14 d). We predicted low and constant ecdysteroid levels, independent of both mating status and time post mating trial—our results support this prediction. Our third experiment explored the relationship between exposure to conspecific courtship and ecdysteroid levels; again, we found no relationship. Thus, circulating ecdysteroid concentrations were not associated with any aspect of reproductive biology we explored. However, we found a negative trend between female age post maturation and circulating ecdysteroid concentration in the species for which we had the greatest age range, consistent with its role as a molting hormone.

Keywords: 20-hydroxyecdysone, female choice, oogenesis, vitellogenesis

An animal's behavior is the result of complex interactions between a variety of internal (e.g., physiological state) and external (e.g., past environmental conditions) factors. In arthropods, hormones, in particular juvenile hormone and ecdysteroids, commonly act as important mediators of such complex interactions. Previous research focusing on a variety of arthropod groups demonstrates that hormone levels can influence aggression, social interactions and dominance status (American lobster *Homarus americanus*: Bolingbroke & Kass-Simon 2001; cockroach *Nauphoeta cinerea*: Kou et al. 2009; paper wasps *Polistes gallicus*: Röseler et al. 1984; bumble bees *Bombus terrestris*: Bloch et al. 2000); sexual receptivity (insects: Ringo 1996); as well as parental care (burying beetles *Nicrophorus* spp.: Scott & Panaitof 2004). Ecdysteroid-regulated behaviors are often associated with particular stages of development. For example, exogenously applied 20-hydroxyecdysone (20E) at late pre-ecdysis causes female lobsters to become more aggressive, which is similar to their natural premolt behavior (Bolingbroke & Kass-Simon 2001).

Despite the vast amount of knowledge that has been accumulated regarding the relationship between hormones and behavior in insects and crustaceans, our knowledge of the function of hormones in arachnids is relatively limited. Non-spider arachnid studies have focused mostly upon ticks (Ixodoidea) (Germond et al 1982; Bouvier et al. 1982) and opilionids (Opilionidae) (Romer & Gnatzy 1981). Recent investigations across various spider families have suggested that ecdysteroids play a role in reproductive physiology and behavior including regulation of ovarian development and vitellogenesis (Trabalon et al. 1998, 1992; Pourié & Trabalon 2003), pheromone production and cannibalistic interactions (Trabalon et al. 1998, 2005) and sexual receptivity (Trabalon et al. 2005). To date, ecdysteroids have been studied in only a handful of spider species: e.g., *Coelotes terrestris* (Wider 1834); *Tegenaria domestica* (Clerck 1757) (Trabalon et al. 1992); *Pisaura mirabilis* (Clerck 1757) (Bonnaric and De Reggi 1977); *T. atrica* (C.L. Koch 1837) (Trabalon et al. 1998) and *Brachypelma albopilosum* Valerio 1980 (Trabalon & Blais 2012).

Here we quantify hemolymph ecdysteroid levels in *Schizocosa* wolf spiders. The adult morphology and associated courtship behavior of male *Schizocosa* vary widely and are often complex, containing both visual ornaments and movements as well as seismic courtship components (reviewed in Stratton 2005; Framenau & Hebets 2007; Vaccaro et al. 2010; Hebets et al. 2013). This genus has been at the forefront of studies of sexual selection via female mate choice, secondary sexual trait evolution and function, and complex signaling (reviewed in Uetz & Roberts 2002; Hebets et al. 2011, 2013; Rundus et al. 2011). Information gained regarding putative mechanistic underpinnings of female reproductive behavior would add significantly to our knowledge of basic reproductive physiology in this well-studied system. Additionally, the genus *Schizocosa* encompasses one of the first arthropod species for which an effect of subadult experience on subsequent mate choice behavior has been demonstrated (Hebets 2003; Hebets & Vink 2007), making species in the genus a good choice for studies addressing potential effects of female experience on reproductive behavior and physiology.

We used three species of *Schizocosa* readily available to us in the laboratory [*Schizocosa avida* (Walckenaer 1837), *S. rovneri* Uetz & Dondale 1979 and *S. uetzi* Stratton 1997] to investigate the relationship between hemolymph ecdysteroid levels and female reproductive behavior and experience. Our

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Table 1.—Collection information for *Schizocosa* species.

	Collection Sites	Collection Time	GPS Coordinates
S. avida	Lancaster CO, NE USA	June & July 2007 & 2008	40.749, −96.817
S. rovneri	Lafeyette CO, MS USA	10–11 April 2010	34.431, −89.706
S. uetzi	Lafeyette CO, MS USA	21–22 May 2007	34.431, −89.706

first experiment tested the hypothesis that female reeeptivity is associated with heightened levels of hemolymph ecdysteroids. Our second experiment tested the hypothesis that hemolymph ecdysteroids remain stable following copulation, despite the likely transition from pre-vitellogenesis to vitellogenesis. Finally, our third experiment explored the possibility that exposure to conspecific male courtship influences hemolymph ecdysteroid levels.

Our first experiment builds upon prior work in other spider groups. Ecdysteroid levels are known to increase sharply in females following their final maturational molt; in *C. terrestris* and *T. domestica*, this happens 10–30 days post maturation (Trabalon et al. 1992). This increase in hemolymph ecdysteroids coincides with the transition between the pre-vitellogeneic and early vitellogeneie phase of oocyte development in virgin females (Trabalon et al. 1992, 1998). This natural increase may also coincide with increased female receptivity, as some females exhibit peak receptivity at ~ 3 weeks post maturation (e.g., *Schizocosa ocreata* (Hentz 1844): Uetz & Norton 2007). Additionally, prior hormone manipulation work is consistent with the hypothesis that an increase in receptivity is associated with increased ecdysteroid levels. Following an injection of 20E in *T. atrica,* females showed an increase in the frequency of sexual receptivity (Trabalon et al. 2005).

Our second experiment aimed to examine the influence of mating on ecdysteroid levels by quantifying hemolymph ecdysteroid levels at various time points post-copulation. We predieted that ecdysteroid levels would be relatively low in mated females and would be independent of time post mating (i.e., levels would remain stable). The maturation of ovaries in spiders occurs in three phases: 1) a pre-vitellogenic phase, 2) a vitellogenic phase, and 3) a post-vitellogenie phase (Andre and Rouiller 1957; Sotelo and Trujillo-Cenoz 1957; Trabalon et al. 1992), with six distinct stages described (Trabalon et al. 1992). Late vitellogenesis and post-vitellogenesis (stages 5–6) have only been observed in mated females and appear to be associated with relatively low and constant hemolymph ecdysteroid levels (Trabalon et al. 1992), and the highest levels of hemolymph ecdysteroids are found in virgin versus mated females (Trabalon et al. 1998).

Our final experiment builds upon previous behavioral work showing that the mate choice of *Schizocosa* females can be altered after exposure to courtship advances from conspecific males (Hebets 2003, Hebets & Vink 2007). *Schizocosa uetzi* was chosen as the focal species for the third experiment, since it is in this species that an effect of subadult experience on adult mate choice was first demonstrated (Hebets 2003). Given the previously demonstrated effect of exogenously injected 20E on sexual receptivity in *T. atrica* (Trabalon et al. 2005), we hypothesized that variation in ecdysteroids underlies this previously observed mate choice preference. If so, we predicted that females exposed to male courtship would have higher levels of hemolymph ecdysteroids than unexposed females.

METHODS

General methods.—All spiders were collected as subadults (Table 1) and were housed individually in visually isolated plastic containers placed together in large plastic tubs filled with water (see Rundus et al. 2011). Spiders were provided crickets (*Acheta domesticus*) twice a week. To the best of our knowledge, all three species of *Schizocosa* used in this study have a one-year life cycle with maturation molts oecurring in late spring/early summer. Egg sacs are laid in mid-summer and overwintering occurs at the juvenile stages.

Experiment 1: Female receptivity and ecdysteroids (*Schizocosa avida*).—We chose *S. avida* as the focal species for this first experiment as it is a relatively large species within the genus (Dondale & Redner 1978), which enabled us to take hemolymph samples from individuals prior to mating trials. All individuals survived our collection technique. This was important because our aim was to examine the relationship between eedysteroid levels and subsequent likelihood to copulate. This speeies was locally abundant and thus readily available. Twenty-eight mature female *S. avida* spiders ranging in age from 3–35 days post-maturation (mean ± SE = 21.9 ± 1.89) were used in receptivity trials. We purposefully included a wide range of ages to examine age-related variation in ecdysteroid levels simultaneously. Females were weighed two days prior to receptivity trials, hemolymph was collected via bleeding (see "Hemolymph Collection") and individuals were returned to their containers. After two days of recovery, females did not display any noticeable aberrant behaviors.

Females were placed in a circular plastic arena (radius: 11.5 cm, height: 7.5 cm) two days post bleeding and were allowed to acclimate and lay silk on a piece of filter paper lining the bottom of the arena. After 2 h of acclimation, a mature conspecific male was introduced. Females and males were only used once. Each pair was allowed to interact for 30 min. We recorded the presence or absence of eopulation.

Experiment 2: Time post-copulation and ecdysteroids (*Schizocosa rovneri*).—Fifty-eight mature virgin female *S. rovneri* ranging in age from 10–39 days post maturation (mean ± SE = 21.4 ± 0.96) were used in mating trials with mature virgin conspecific males. We had no a priori reason to use *S. rovneri* for this experiment, but took advantage of their availability in the laboratory due to other ongoing studies. Individual males and females were only used once. Our design enabled us to determine whether hemolymph ecdysteroid levels would change following copulation, as we compared these levels between mated and unmated females at three time points post mating trials (and thus, post eopulation for mated females). Mating trials were run in cireular arenas exactly as in Experiment 1. If copulation occurred, the pair was left

undisturbed until it ended. If no copulation occurred, the female was returned to her home container. We collected hemolymph from females (mated and unmated) at three randomly assigned time points following mating trials: 24 h, 7 d or 14 d. We collected hemolymph only onee from each female. All females were weighed before bleeding and bleeding occurred at the same time of day as the original mating trials ±2 h (see "Hemolymph Collection").

Of the 58 females used in this experiment, 29 copulated. We collected hemolymph from 15 females at 24 h post mating trial (copulation = 5, no copulation = 10); from 17 females at 7 d post mating trial (copulation = 8, no copulation = 9) and from 26 females at 14 d post mating trial (copulation = 16, no copulation = 10).

Experiment 3: Courtship exposure and ecdysteroids (*Schizocosa uetzi*).—To explore the relationship between exposure to male courtship and hemolymph ecdysteroids, we compared ecdysteroid levels of virgin females exposed versus unexposed to male courtship. Since subadult female *S. uetzi* respond to exposure to eonspeeific male courtship (Hebets 2003), we chose this as our focal species. Forty-six mature females ranging in age from 35–55 days post maturation molt (mean ± SE = 42.9 ± 0.69) were used.

Females were assigned to one of two treatments: 1) exposure to conspecific male courtship (exposed; n = 30) or 2) no exposure to conspeeific male courtship (unexposed; n = 16). At the start of a trial, a female was allowed to acclimate for 15 min in a plastic arena (8.8 cm × 8.8 cm × 11 em) lined with filter paper to enable seismic cue transmission. The arena had its sides covered with masking tape to provide visual isolation from the surrounding room. For exposed females, following the acclimation period a thin circular transparent acetate barrier (radius: 3 cm, height: 5 cm) was lowered around the female, enclosing her in the center of the larger arena. A mature conspecific male was then placed in the surrounding arena, and the pair was observed for 30 min. During this period females were exposed to male visual and seismic courtship signals, but could not contact the males. For unexposed females, an identical thin transparent acetate barrier was lowered after the 15 min acclimation period, enclosing the female for 30 min, but no male was introduced in the larger arena. Females were bled immediately following their trial; no more than 5 min passed between trial ending and hemolymph collection (see "Hemolymph Collection").

Hemolymph collection.—Each spider was weighed and placed in a quart-sized Ziploc plastic bag that had one corner cut. Spiders were positioned in the bag such that one leg could protrude through the opening in the cut corner. This leg was then cut in the middle of the femur and hemolymph was collected in glass micropipettes. If 10 µL could not be obtained from the first cut, a second leg was cut. In some cases less than 10 µL of hemolymph were colleeted. Hemolymph samples were blown into microcentrifuge tubes containing 300 µL of 90% methanol. Tubes were vortexed for ~5 s and stored in a −20 °C freezer until ecdysteroids were assayed.

Radioimmunoassay analysis.—Total ecdysteroid concentration was estimated from three replicates of each hemolymph sample using a standard radioimmunoassay similar to that described in Zera and Bottsford (2001). 20E was used as the standard. Briefly, a small sample of hemolymph extract or ecdysteroid standard was added to a test tube, solvent was evaporated under a gentle stream of nitrogen and 100 µL of diluted anti-ecdysteroid polyclonal antiserum in 0.1 M borate buffer containing 75 mM NaCl (pH 8.3) was added. The antiserum had been produced in the laboratory of W. E. Bollinbacher (University of North Carolina, Chapel Hill) and was provided by E. S. Chang (Bodega Marine Laboratory, Bodega Bay, California). Subsequently, 5000 CPM ecdysone (Ecdysone α-[23,24-^{3}H(N)], PerkinElmer Inc., Boston, MA) in 100 µL borate buffer was added. Tubes were vortexed for ~5 s and incubated overnight at 4 °C.

The next day, 50 µL of a 10 mg/mL bovine gamma globulin solution (co-precipitant) in borate buffer was added to each tube, followed by 250 µL of saturated aqueous ammonium sulfate ($(NH_4)_2SO_4$) to precipitate the antibody-ecdysteroid complex. Tubes were vortexed for ~5 s, incubated on ice for 30 min and centrifuged at 5000 RPM for 10 min at 4 °C. The supernatant was removed and 250 µL of 50% saturated $(NH_4)_2SO_4$ in borate buffer was added to each tube, which was vortexed and centrifuged as above. Both sets of supernatants were discarded and the pellets (containing antibody plus bound ecdysteroid) were dissolved in 500 µL of borate buffer, transferred to plastic scintillation vials and counted on a 1450 MicroBeta TriLux Liquid Scintillation Counter (PerkinElmer, Boston, MA). Ecdysteroid concentrations in hemolymph samples were estimated by standard nonlinear regression using Prism GraphPad Version 4. Standard curves were constructed from the following 20E masses (0.02, 0.07, 0.2, 0.7 and 2 ng), all of which gave r^2 values greater than 0.99 with the exception of one at r^2 = 0.9661. Back calculations were used to determine the ecdysteroid concentration from the interpolated masses in hemolymph samples based on standard curves generated during each assay.

Statistical analyses.—For all three experiments we examined the relationship between our predictor variables (treatment and age) and the response variable (measured concentration of ecdysteroids), using least squares regression analyses. In all instances we first ran a full model with all possible interactions and included female weight as a predictor variable. We found no significant interactions and no effect of female weight and thus we report only our reduced models. All analyses were eonducted in JMP (Version 8). To conform to assumptions of normality, we used a natural log (ln) transformation of our response variable, ecdysteroid concentration.

Effect sizes were calculated using from the P-values of our models with the following website: http://www.campbellcollaboration.org/resources/effect_size_input.php.

RESULTS

None of our least squares regression models were significant. Receptivity did not predict ecdysteroid levels in *S. avida* ($F_{3,21}$ = 1.84, P = 0.17, Fig. 1). Neither mating status (copulated vs. virgin), time post mating trial, nor an interaction between the two, predicted ecdysteroid levels in *S. rovneri* ($F_{6,51}$ = 0.72, P = 0.64, Fig. 2). Experience with a conspecific male in *S. uetzi* had no influence on hemolymph ecdysteroid levels ($F_{3,42}$ = 0.64, P = 0.59, Fig. 3). Ultimately, we found no evidence of a relationship between our measured concentration of ecdysteroids in the hemolymph and any of our examined aspects of reproductive behavior. Details of

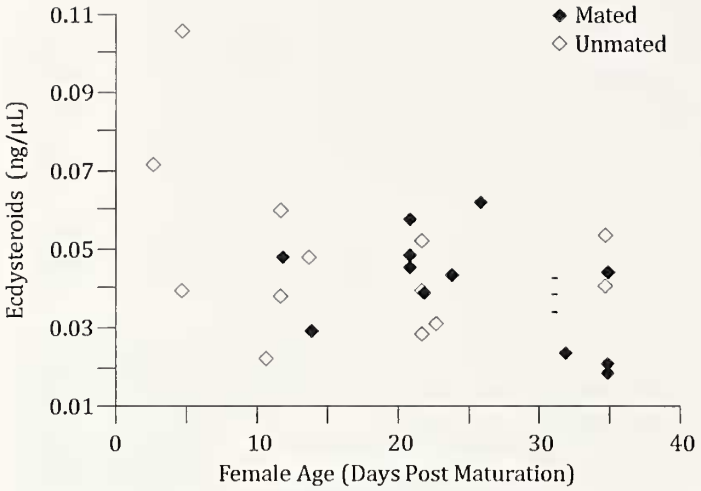

Figure 1.—Hemolymph ecdysteroid concentrations of *S. avida* females of different ages. There was a negative trend of decreasing ecdysteroid concentration with increasing age ($P = 0.05$). This trend was largely driven by a single female. The three dashes represent individuals whose copulation status was not tested.

each of our models can be seen in Table 2. Younger *S. avida* females had ecdysteroid levels that were nearly significantly higher than those of older females (see Table 2; $r^2 = 0.20$, $P = 0.05$, Fig. 1). A single female appeared to be largely responsible for this near significance (Fig. 1).

DISCUSSION

This study reports the first naturally occurring levels of ecdysteroids in the hemolymph of *Schizocosa* wolf spiders. Observed levels were comparable to concentrations detected previously using enzyme immunoassay in adult spiders of *Tegenaria domestica* (0.00441–0.01767 ng/µL), *Coelotes terrestris* (0.00071–0.02528 ng/µL) (Trabalon et al. 1992), *Tegenaria atrica* (0.00747–0.01345 ng/µL) (Trabalon et al. 1998) and

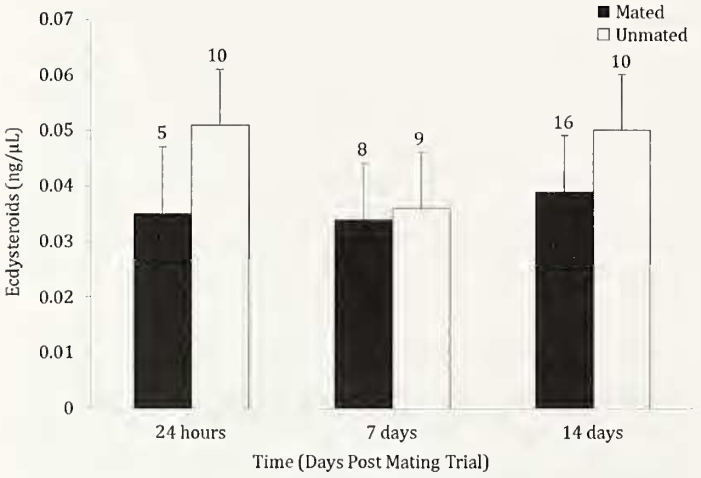

Figure 2.—Comparison of hemolymph ecdysteroid concentrations in *S. rovneri* mated versus unmated females at 24 h, 7 d and 14 d after mating trials. The bars represent the average hemolymph ecdysteroid concentration, and the error bars represent the standard error. There was no significant difference in ecdysteroid concentration between mated and unmated females ($P = 0.21$) or time post trial ($P = 0.11$). The numbers above the bars indicate sample sizes.

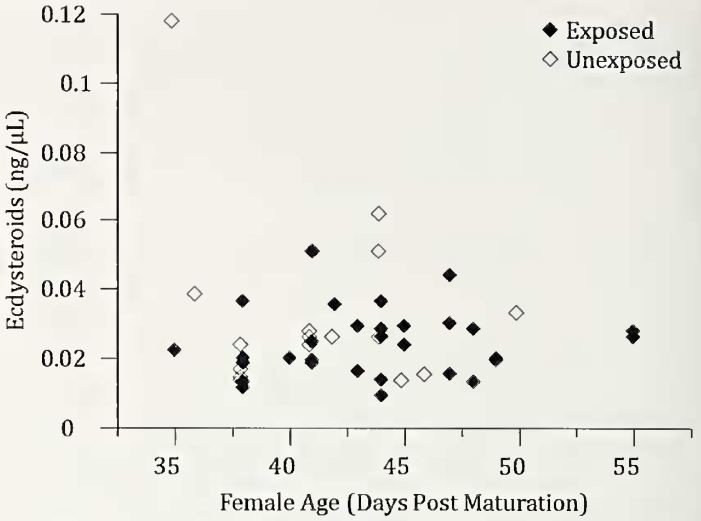

Figure 3.—Hemolymph ecdysteroid concentrations in *S. uetzi* females exposed or unexposed to conspecific male courtship. There was no significant difference in ecdysteroid concentration between courtship treatment (exposed versus unexposed) ($P = 0.37$), age ($P = 0.85$), or the interaction of these variables ($P = 0.41$).

Brachypelma albopilosum (females: 0.00744 ng/µL, males: 0.01122 ng/µL) (Trabalon & Blais 2012). We found no relationship between ecdysteroids and reproductive behavior in the *Schizocosa* species tested. Specifically, we found no relationship with 1) likelihood to copulate (i.e., female receptivity) (Exp. 1 – *Schizocosa avida*); 2) time post copulation (Exp. 2 – *Schizocosa rovneri*) or 3) female exposure to conspecific courtship (Exp. 3 – *Schizocosa uetzi*). Our calculated effect sizes (denoted *r*, Table 2) were not particularly large, indicating that our lack of significance was probably not attributable to low sample sizes.

In our first experiment, we predicted higher levels of hemolymph ecdysteroids in receptive versus unreceptive *S. avida* females, but we found no indication that receptivity was a good predictor of hemolymph ecdysteroid levels. Our expectations were based upon prior work in other spiders showing high ecdysteroid levels 10–30 days post maturation and in 20 day old virgin females (Trabalon et al. 1992, 1998)—a time during which *Schizocosa* females are known to show peak receptivity (Uetz & Norton 2007). In contrast to our prediction, our results indicate a trend towards an immediate decrease in hemolymph ecdysteroids following maturation (Fig. 1), consistent with a role in molting. Regardless, we did not detect a surge in hemolymph ecdysteroids in virgin females 10–30 days post maturation. It is important to note that in addition to the fact that *Schizocosa* wolf spiders (Family Lycosidae) are not close relatives of the previously studied spider species (e.g., *Coelotes*, Family Amaurobiidae; *Tegenaria*, Family Agelenidae), the method of collecting hemolymph samples also varied across studies. In *C. terrestris*, *T. domestica*, and *T. atrica*, hemolymph was collected from the thorax with glass microcapillary tubes. In contrast, our hemolymph (present study) was collected in glass microcapillary tubes from clipped walking legs. Given the proximity of the hemolymph collection site in the prior studies to the central nervous system of the spider, it is possible that ecdysteroid levels in the prosoma (a potential location for

Table 2.—Mean hemolymph ecdysteroid levels for all species and results of least squares regression models. Experiment 1 compared ecdysteroid levels between females that did and did not copulate. Experiment 2 compared ecdysteroid levels between females that did and did not copulate across time points. Experiment 3 compared ecdysteroid levels between females exposed and unexposed to male courtship.

Experiment	Ecdysteroid Mean ± SE (ng/μL)	Effect	F Ratio	P value	*r*	CI
Exp. 1 (*S. avida*)	0.04 ± 0.003	Female age	4.29	0.05	0.39	−0.003–0.66
		Copulation (yes/no)	0.01	0.91	0.02	0.37–0.42
		Age * Copulation	0.73	0.4	0.18	−0.24–0.53
Exp. 2 (*S. rovneri*)	0.042 ± 003	Female age	0.49	0.49	0.09	−0.17–0.34
		Copulation (yes/no)	2.48	0.12	0.21	−0.05–0.44
		Time Treatment	0.85	0.43	0.11	−0.16–0.35
Exp. 3 (*S. uetzi*)	0.027 ± 0.002.5	Female age	0.035	0.85	0.03	−0.26–0.32
		Courtship Treatment	0.82	0.37	0.14	−0.16–0.41
		Age * Courtship	0.69	0.41	0.12	−0.17–0.40

ecdysteroid synthesis) are not equivalent to levels circulating in the walking legs. Additionally, although prior work in other species clearly demonstrated an increase in ecdysteroid concentrations 10–30 days post maturation, this was correlated with timing of oocyte development, specifically with the transition between pre-vitellogenesis and early vitellogenesis (see below). In *Schizocosa*, the timing of this transition is currently unknown. Finally, the strongest evidence for a link between receptivity and ecdysteroid levels comes from a hormone manipulation study. In *T. atrica,* females were injected with 2 ng of 20E in 1 μL of Ringer's solution and showed an increase in reeeptivity. Such a concentration was expected to result in a hemolymph concentration of 0.5 ng/μL, which is much higher than that found in unmanipulated individuals (Trabalon et al. 2005). This large dose of hormone was presumed necessary, as 20E is rapidly degraded in other spiders (Connat et al. 1988). However, the unnaturally high hormone dose administered may have caused the observed behavioral changes in these spiders. Incidental (non-physiological) side-effects of hormone injection are not uncommon in hormone studies of invertebrates (Zera 2007). Given the results of the previous hormone manipulation study and the suggestion of a relationship between 20E and receptivity in *T. atrica,* this species would be ideal for directly testing the hypothesis that circulating ecdysteroid levels are correlated with receptivity behavior.

In spiders, vitellogenesis does not occur until females have copulated, yet Pourié & Trabalon (2003) were able to induce vitellogenesis in virgin females through injections of 20E, suggesting its role in vitellogenesis. However, all assays of hemolymph ecdysteroid levels in mated females indicate that ecdysteroid levels are constant and low following copulation (Trabalon et al. 1992, 1998). The results of our second experiment are consistent with these earlier findings, as our measured ecdysteroid levels in *S. rovneri* did not vary according to their mating status (mated versus virgin), their time post mating trial (24 h, 7 d, 14 d) or an interaction between the two (Fig. 2). One potential explanation for these results is that ecdysteroids are not involved in vitellogenesis in these spiders. This would not be unprecedented, as not all insects use ecdysteroids to initiate vitellogenesis [e.g., lubber grasshopper *Romalea microptera* (Hatle et al. 2003); cockroach *Leucophaea maderae* (Engelmann 2002)]. Alternatively, and potentially more likely, levels of ecdysteroids circulating in the hemolymph of the walking legs may not be the most relevant measures for their role in vitellogenesis. In insects, ecdysteroidogenesis occurs in the gonads, and in non-insect arthropods this process occurs in specialized tissues or organs (e.g., the Y-organ in crustaceans) and ecdysteroids are trafficked to the gonad via lipoproteins such as vitellogenin (Brown et al 2009). Thus, measurement of ovarian 20E concentrations may reveal differences between mated and non-mated females.

Finally, our last experiment was an exploratory endeavor to determine whether there might be a relationship between a female's prior experience with a courting conspecific male and ecdysteroid levels. Given that we found no relationship between female receptivity and ecdysteroid levels (Exp. 1), it is not surprising that we similarly failed to find a relationship in this third experiment (Fig. 3). Arguably, our exposures were conducted on relatively old mature virgin females so the possibility remains that exposure earlier in life influences ecdysteroid levels. Further, our assays focused solely on ecdysteroid levels in the hemolymph and, as above, the possibility remains that concentration changes take place in specific tissues or organs, such as the ovary.

Early investigations in spiders implicated ecdysteroids as molting hormones (reviewed in Krishnakumaran & Schneiderman 1970; Bonaric & Reggi 1977; Bonaric 1987), and our results are consistent with this function. In many arthropod groups, the level of 20E is known to fluetuate over a molt cycle in a predictable pattern [e.g., crustaceans: shore crab *Carcinus maenas* (Styrishave et al. 2008); prawn *Macrobrachium rosenbergii* (Okumura & Aida 2000); isopod (*Armadillidium vulgare*) Suzuki et al. 1996; lobster, *Homarus americanus* Synder & Chang 1991); several orders of insects: termite *Coptotermes formosanus*: Isoptera (Raina et al. 2008); beetle *Zophobas atratus*: Coleoptera (Delbecque et al. 1997); fruit fly *Drosophila melanogaster*: Diptera (Handler 1982); arachnids: tick *Ornithodoros moubata* (Germond et al. 1982); spiders *Pisaura mirabilis* (Bonaric & Reggi 1977; Bonaric 1987). Typically, ecdysis, or the final shedding of the cuticle, occurs shortly after 20E concentration begins to decrease from its peak concentration, ultimately returning to its baseline level. This is a similar pattern to what we observed in *S. avida,* the only species for which we have data on females immediately following their maturation molt.

In summary, we quantified the concentration of ecdysteroids in three species of *Schizocosa* wolf spiders encompassing a variety of reproductive behaviors. We found no evidence for a relationship between hemolymph ecdysteroid levels and

female reproductive behavior. Given that ecdysteroid synthesis and secretion are often organ-specific in other arthropod groups, however, it is possible that our measures of circulating hemolymph ecdysteroids did not capture ecdysteroid concentrations pertinent to our focal reproductive behavior. Our results are consistent with ecdysteroids acting as the molting hormone in these spiders, but suggest that circulating levels do not act significantly in female mating behavior or reproductive physiology.

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